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Hes1 and *Hes5* as Notch effectors in mammalian neuronal differentiation

Toshiyuki Ohtsuka[1,2], Makoto Ishibashi[2–5], Gerald Gradwohl[6], Shigetada Nakanishi[2], François Guillemot[6,7] and Ryoichiro Kageyama[1,2,7]

[1]Institute for Virus Research, Kyoto University, Shogoin-Kawahara, Sakyo-ku, Kyoto 606-8507, Departments of [2]Biological Sciences and [3]Anatomy, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606-8501, [4]Inheritance and Variation, PRESTO, Japan Science and Technology Corporation, Kyoto 606-8501, Japan and [6]Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ Universite Louis Pasteur/College de France, Illkirch, CU de Strasbourg, France

[5]Present address: Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

[7]Corresponding authors
e-mail: rkageyam@virus.kyoto-u.ac.jp

T.Ohtsuka, M.Ishibashi and G.Gradwohl contributed equally to this work

While the transmembrane protein Notch plays an important role in various aspects of development, and diseases including tumors and neurological disorders, the intracellular pathway of mammalian Notch remains very elusive. To understand the intracellular pathway of mammalian Notch, the role of the bHLH genes *Hes1* and *Hes5* (mammalian *hairy* and *Enhancer-of-split* homologues) was examined by retrovirally misexpressing the constitutively active form of Notch (caNotch) in neural precursor cells prepared from wild-type, *Hes1*-null, *Hes5*-null and *Hes1-Hes5* double-null mouse embryos. We found that caNotch, which induced the endogenous *Hes1* and *Hes5* expression, inhibited neuronal differentiation in the wild-type, *Hes1*-null and *Hes5*-null background, but not in the *Hes1-Hes5* double-null background. These results demonstrate that *Hes1* and *Hes5* are essential Notch effectors in regulation of mammalian neuronal differentiation.

Keywords: bHLH/*Hes1*/*Hes5*/neuronal differentiation/ Notch

Introduction

Activation of the transmembrane protein Notch inhibits cellular differentiation, and this process enables the maintenance of progenitors and the later response to different inductive cues, thereby generating cell-type diversity (Artavanis-Tsakonas *et al*., 1995; Lewis, 1996; Dorsky *et al*., 1997; Weinmaster, 1997). In mammals, Notch is involved in differentiation of a variety of cell types including neurons and blood cells, and also in various diseases such as tumors and hereditary neurological disorders (Ellisen *et al*., 1991; Jhappan *et al*., 1992; Kopan *et al*., 1994; Nye *et al*., 1994; Joutel *et al*., 1996; Milner *et al*., 1996; Pear *et al*., 1996; Robey *et al*., 1996; Capobianco *et al*., 1997; de Angelis *et al*., 1997; Gridley, 1997). While Notch plays an important role in such various aspects of mammalian development and diseases, its intracellular pathway remains very elusive.

Notch is processed by a furin-like protease and is present as a heterodimeric molecule at the cell surface (Blaumueller *et al*., 1997; Logeat *et al*., 1998). When Notch is activated by its ligand, the intracellular domain of Notch (ICN) is likely to be cleaved and translocate into the nucleus (Kopan *et al*., 1996; Luo *et al*., 1997; Pan and Rubin, 1997; Lecourtois and Schweisguth, 1998; Schroeter *et al*., 1998; Struhl and Adachi, 1998). In the nucleus, ICN forms a complex with the DNA-binding protein RBP-J (mouse)/Suppressor of Hairless (*Drosophila*) and activates gene expression (Artavanis-Tsakonas *et al*., 1995; Honjo, 1996). The basic helix–loop–helix (bHLH) genes *Hes1* and *Hes5* (mammalian *hairy* and *Enhancer-of-split* homologues 1 and 5) are candidate target genes for mammalian Notch pathway (Kageyama and Nakanishi, 1997) on the grounds that the constitutively active form of Notch (caNotch) can not only activate *Hes1* and *Hes5* promoters through the RBP-J-binding sites in a co-transfection study, but also induce the endogenous *Hes1* expression (Jarriault *et al*., 1995; Hsieh *et al*., 1997; Nishimura *et al*., 1998), and that *Hes1*, like caNotch, inhibits neuronal and muscle differentiation (Sasai *et al*., 1992; Ishibashi *et al*., 1994). In addition, recent data showed that treatment with the Notch ligand Delta also induces the endogenous expression of *Hes1* or *Hes5* in neighboring cells (Jarriault *et al*., 1998; Wang *et al*., 1998). However, the functional linkage between Notch and *Hes* is still hypothetical, and it remains to be determined whether *Hes1* and *Hes5* are functionally required for the Notch signaling.

In order to investigate the role of *Hes1* and *Hes5* in the Notch pathway, we generated *Hes1-Hes5* mutant mice. We found that *Hes5* mutation, like *Hes1* mutation (Ishibashi *et al*., 1995; Tomita *et al*., 1996a), led to premature neuronal differentiation, and that in the *Hes1-Hes5* double mutation, the severity of premature differentiation was enhanced, indicating the functional redundancy of the two *Hes* genes. Retinal explant cultures or neural precursor cell cultures were also prepared from the wild-type and mutant embryos; these cultures were infected with retrovirus that directed expression of caNotch. We found that caNotch inhibited neuronal differentiation of wild-type, *Hes1*-null and *Hes5*-null cells, but not of *Hes1-Hes5* double-null cells. These results demonstrate that *Hes1* and *Hes5* functionally compensate each other, and together are essential for Notch activity in regulation of mammalian neuronal differentiation.

Results

Construction of caNotch-transducing retrovirus

In order to examine the effects of misexpression of caNotch, we generated two retroviruses: caNotch-alkaline phosphatase (AP) and C-AP (Figure 1A). The former virus directed expression of caNotch that consisted of the transmembrane region, the RAM23 domain known to interact with RBP-J (Tamura *et al*., 1995), the cdc10/ankyrin repeats, and the nuclear localization signal (Figure 1A). The Flag epitope was fused at the N-terminus to monitor caNotch expression (Figure 1A). The other virus, C-AP, was a negative control virus directing expression of a further truncated form of Notch that lacked essential regions such as the RAM23 domain and cdc10/ankyrin repeats (Figure 1A). Both viruses directed through the IRES sequence to express human placental AP, which is advantageous for determining cell types because of its membrane association (Figure 1A). caNotch-AP virus successfully directed co-expression of AP and caNotch, as evidenced by Flag epitope co-expression (Figure 1C and D).

caNotch inhibits neuronal differentiation

Retinal explant cultures prepared from wild-type embryos at day 16.5 (E16.5) or neural precursor cell cultures prepared from wild-type brains at E10.5 were infected with the retroviruses. Two weeks later, the fates of virus-infected cells were determined by detecting AP expression. During this culture period, rod photoreceptors in the outer nuclear layer (ONL) and bipolar interneurons in the inner nuclear layer (INL) are the major neuronal types generated in the retina (Turner and Cepko, 1987). Cells infected with C-AP virus differentiated normally into mature neurons such as rods and bipolar cells in the explant cultures (Figure 1B). These virus-infected cells were present as single cells or clusters consisting of one or two rods and a bipolar cell (Figure 1B). In contrast, cells infected with caNotch-AP virus grew abnormally, and formed large clusters consisting of at least 10 and up to 20 cells in the retina (Figure 1C and D), as reported previously (Bao and Cepko, 1997). While most of the cells infected with caNotch-AP were located in the ONL of the retina (Figure 1C and E, stained red), which contains rods, these virus-infected cells were negative for the rod marker rhodopsin (Figure 1E, green). In addition, these virus-infected cells were negative for other neuronal markers such as neurofilament, protein kinase C and calbindin (data not shown), suggesting that they were inhibited from differentiating into neurons. Furthermore, many of the caNotch-AP virus-infected cells incorporated bromodeoxyuridine (BrdU) (Figure 1F and G), indicating that they were still undergoing mitosis.

Similarly, in neural precursor cell cultures prepared from embryonal brains, cells infected with caNotch-AP virus grew abnormally and formed large clusters without extending neurites after 14 days in culture (Figure 1K and M, red). These caNotch-expressing cells did not express the neuronal marker MAP2, in contrast to the surrounding MAP2$^+$ non-infected cells, which extended neurites (Figure 1L and M, green). These results indicate that caNotch-expressing cells were inhibited from differentiating into neurons (Table I). In contrast, cells infected with C-AP virus were present as single cells (data not shown) or formed clusters consisting of cells that extended neurites (Figure 1H and J, red or yellow) and expressed MAP2 (Figure 1I and J, green or yellow). Thus, C-AP virus-infected cells differentiated into neurons (Table I).

caNotch induces the endogenous Hes1 and Hes5 expression

Previous data indicate that *Hes1* and *Hes5* promoters contain RBP-J-binding sites and respond to Notch signaling in transient co-transfection analysis (Jarriault *et al*., 1995; Nishimura *et al*., 1998). However, in some cell types, upregulation of endogenous *Hes* genes was not observed (Shawber *et al*., 1996). We therefore determined whether neural cells infected with caNotch-AP expressed endogenous *Hes1* and *Hes5*. Retinal explant cultures prepared from wild-type embryos were infected with caNotch-AP, and after 2 weeks, endogenous *Hes1* and *Hes5* expression was examined by *in situ* hybridization. Cells infected with caNotch-AP formed large clusters (Figure 2A and C, brown) and expressed both *Hes1* and *Hes5* (Figure 2B and D, arrows), in contrast to the surrounding non-infected cells that expressed no, or only a very low level of, *Hes1* and *Hes5*. These results demonstrate that activation of the Notch pathway induced the endogenous *Hes1* and *Hes5* expression in the retina.

Neural precursor cells were also infected with caNotch-AP, and endogenous *Hes1* and *Hes5* expression was examined by Northern blot experiments. Because only part of the cells were infected with the virus, endogenous *Hes1* and *Hes5* expression was only weakly upregulated by caNotch (Figure 2E). Expression of other *Hes* genes was not detectable in these cultures (data not shown). These results support the hypothesis that *Hes1* and/or *Hes5* mediate the Notch signaling in the nervous system.

Premature neuronal differentiation of Hes mutant brains

To determine whether *Hes1* and/or *Hes5* are functionally required for the Notch pathway, we used *Hes1* mutant mice (Ishibashi *et al*., 1995) and generated *Hes5* mutant and *Hes1-Hes5* double-mutant mice (E.Cau, G.Gradwohl, R.Kageyama and F.Guillemot, unpublished data; see Materials and methods). *Hes5* mutant mice showed apparently normal morphology, suggesting that the functions of *Hes5* may be mostly compensated by other genes. In contrast, *Hes1* mutation leads to premature neuronal differentiation and concomitant brain and eye defects (Ishibashi *et al*., 1995; Tomita *et al*., 1996a). *Hes1-Hes5* double-mutant embryos showed more severe phenotypes, such as earlier lethality and a smaller body size, than *Hes1* single-mutant embryos, indicating that *Hes1* and *Hes5* may compensate each other (E.Cau, G.Gradwohl, R.Kageyama and F.Guillemot, unpublished data). Since premature neuronal differentiation could deplete dividing neural precursor cells, which may hamper the retroviral infection study, the mutant brains were examined immunohistochemically.

At E10.5, the forebrain of the wild-type embryos mostly consisted of nestin$^+$ neural precursor cells (Figure 3A), and only a small population of cells became MAP2$^+$ neurons at the pial surface (Figure 3E and I). In contrast, in the forebrain of *Hes1*-null and *Hes5*-null embryos more

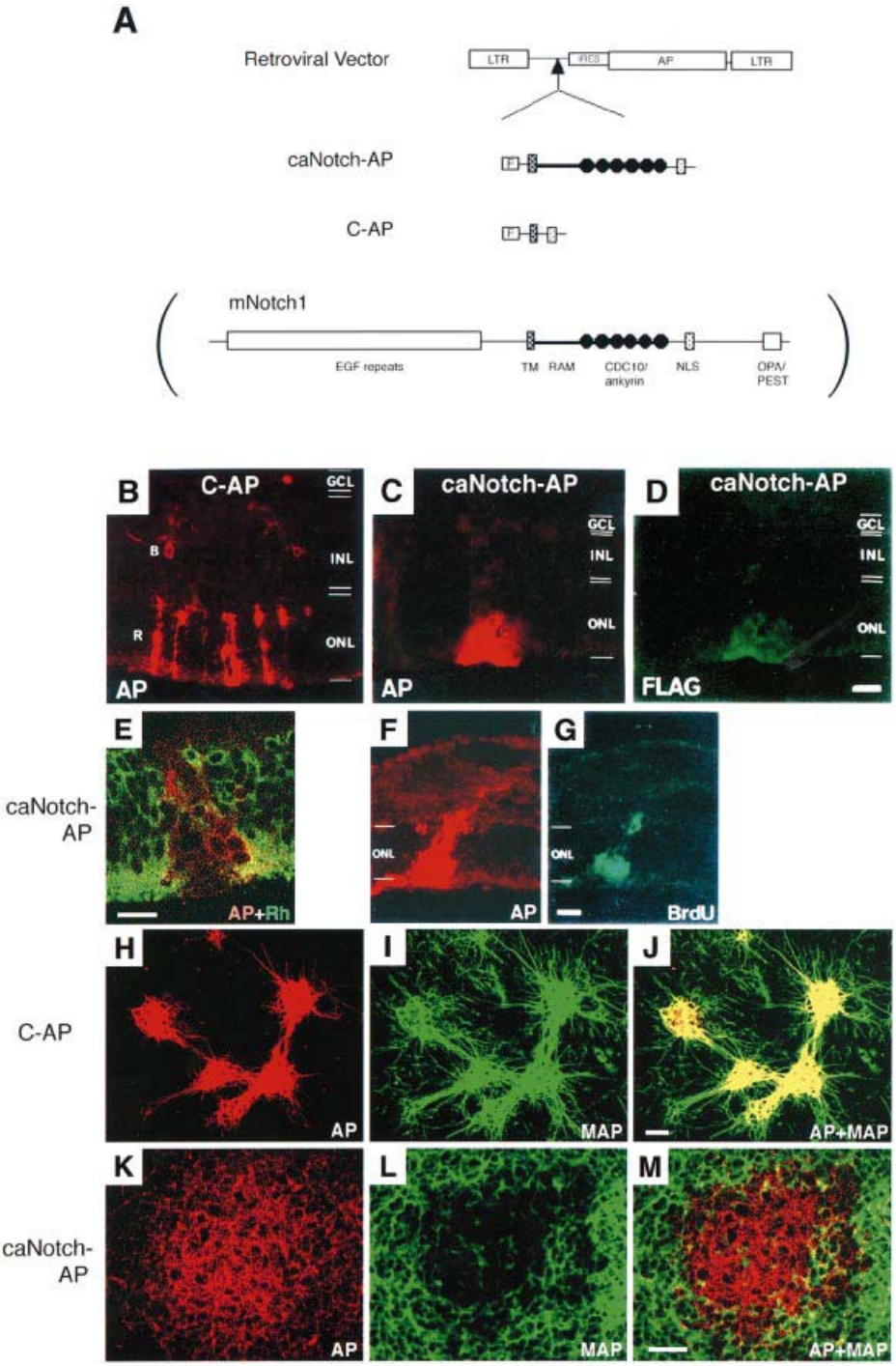

Fig. 1. Retroviral vectors and virus-infection with wild-type cells. (**A**) Schematic structure of the caNotch-AP and C-AP retroviruses. caNotch-AP contains the Flag tag (F), transmembrane region (TM), RAM domain, cdc10/ankyrin repeats and nuclear localization signal (NLS). C-AP contains a further truncated Notch fragment and functions as a negative control. Both viruses direct human AP expression through the IRES sequence. Retinal explant (B–G) and neural precursor cell cultures (H–M) were prepared from wild-type embryos and infected with C-AP (B, H–J) and caNotch-AP (C–G, K–M). (**B**) The retinal explant infected with C-AP was stained with antibody against AP. The virus-infected cells differentiated into rods (R) in the ONL and bipolar cells (B) in the INL. GCL, ganglion cell layer. (C and D) The retinal explant infected with caNotch-AP was stained with antibodies against AP (**C**) and the Flag epitope (**D**). These virus-infected cells formed a cluster and did not differentiate into mature neurons. (**E**) The retinal explant infected with caNotch-AP was stained with antibodies against AP (red) and rhodopsin (Rh, green). The virus-infected cells did not express rhodopsin. (**F** and **G**) BrdU was added to the retinal explant infected with caNotch-AP for the last two days of culture. The explant was stained with antibodies against AP [(F), red] and BrdU [(G), green]. Many caNotch-expressing cells incorporated BrdU, suggesting that these cells were still in a mitotic phase. (**H**–**J**) Neural precursor cell cultures infected with C-AP were stained with antibodies against AP [(H) and (J), red or yellow] and MAP2 [(I) and (J), green or yellow]. These virus-infected cells differentiated into neurons with multiple neurites. (**K**–**M**) Neural precursor cell cultures infected with caNotch-AP were stained with antibodies against AP [(K) and (M), red] and MAP2 [(L) and (M), green]. These virus-infected cells grew abnormally and did not differentiate into neurons. Scale bar, 25 μm.

Table I. Neuronal differentiation of virus-infected cells

Virus	Genotype	AP^+ clusters[a]	$MAP2^+$ clusters[b]	Ratio of neurons[a] (%)
C-AP	wt	366	38	10.4
caNotch-AP	wt	341	2 (0)	0.6
caNotch-AP	*Hes1*$^{-/-}$	70	0 (0)	0
caNotch-AP	*Hes5*$^{-/-}$	189	0 (0)	0
C-AP	*Hes1*$^{-/-}$-*Hes5*$^{-/-}$	59	5	8.5
caNotch-AP	*Hes1*$^{-/-}$-*Hes5*$^{-/-}$	241	17 (15)	7.1

[a]Each isolated single AP^+ cell was also counted as a cluster. The neural precursor cell cultures contained many mesenchymal cells which were also infected with virus. Because of the difficulty to distinguish between neural and mesenchymal cells in some cases, the number of all infected clusters was counted. As a result, the ratios of neurons were lower than those observed when only neural precursor cells were used.
[b]$MAP2^+$ and AP^+ clusters were counted. The total number of isolated single cells and small clusters (2–10 cells) is indicated in parentheses.

$MAP2^+$ neurons were present (Figure 3F and G) and some of them seemed to express MAP2 prematurely, before reaching the pial surface (Figure 3J and K). Thus, although the morphology of *Hes5*-null embryos appeared normal, neuronal differentiation was accelerated, indicating that *Hes5*, like *Hes1*, also prevents premature differentiation. In the double-mutants, neurons appeared at even greater density (Figure 3H and L), indicating that differentiation is further accelerated in the absence of both *Hes* genes. In addition, the double-mutant brains were smaller and severely deformed (Figure 3D and H). However, in spite of accelerated neurogenesis, many nestin$^+$ neural precursor cells still remained in these mutant brains (Figure 3B–D).

We also examined the retinas of mutant mice. Whereas *Hes1*-null retinas showed premature neuronal differentiation and disruption of the laminar structures (Tomita *et al*., 1996a), *Hes5*-null retinas did not show any apparent abnormality (data not shown). Most of the *Hes1-Hes5* double-mutant embryos died by E12.5, and therefore it was not possible to examine the retinas.

caNotch inhibits neuronal differentiation in the absence of Hes1

To investigate whether *Hes1* is required for the Notch function, retinal explant and neural precursor cell cultures prepared from *Hes1*-null embryos were infected with caNotch-AP virus, and the fates of virus-infected cells were determined.

When infected with caNotch-AP, *Hes1*-null retinal cells formed clusters consisting of many labeled cells (Figure 4A–C, red). In addition, these infected cells did not express mature neuronal markers such as rhodopsin (Figure 4A, green), but expressed the progenitor-specific marker nestin (Figure 4B, green). Furthermore, many of the virus-infected cells incorporated BrdU (Figure 4C, green), indicating that they were still proliferating. Thus, caNotch inhibited retinal neuronal differentiation in the absence of *Hes1*.

Neural precursor cell cultures prepared from E10.5 *Hes1*-null brains were also infected with caNotch-AP. These virus-infected cells formed large clusters (Figure 4D and F, red) and did not express the neuronal marker MAP2, or extend neurites (Figure 4E and F). In contrast, the surrounding non-infected cells expressed MAP2 and extended neurites (Figure 4E and F, green). Thus, these virus-infected cells were inhibited from differentiating into neurons (Table I). These results clearly demonstrate that caNotch can inhibit neuronal differentiation in the absence of *Hes1*.

caNotch inhibits neuronal differentiation in the absence of Hes5

To investigate whether *Hes5* is required for Notch-induced inhibition of neuronal differentiation, *Hes5*-null retinal explant and neural precursor cell cultures were infected with the caNotch-AP virus, and the fates of virus-infected cells were determined. In the retinas prepared from *Hes5*-null embryos, cells infected with caNotch-AP formed large clusters consisting of many labeled cells in the ONL (Figure 5A), and these cells were negative for rhodopsin (Figure 5B, arrowhead). Thus, caNotch inhibited retinal neuronal differentiation in the absence of *Hes5*. In neural precursor cell cultures prepared from *Hes5*-null embryos, cells infected with caNotch-AP formed large clusters and did not express MAP2 or extend neurites (Figure 5C and E, red). In contrast, the surrounding non-infected cells expressed MAP2 and extended neurites (Figure 5D and E, green). Thus, caNotch-AP-infected cells were inhibited from differentiating into neurons (Table I). These results demonstrate that caNotch can inhibit neuronal differentiation in the absence of *Hes5*.

caNotch fails to inhibit neuronal differentiation in the absence of Hes1 and Hes5

Because *Hes1* and *Hes5* could compensate each other in the Notch pathway, we next used *Hes1-Hes5* double-mutant mice to determine whether Notch can function in the absence of both *Hes* genes. Neural precursor cell cultures were prepared from brains of double-mutant embryos and infected with caNotch-AP virus. In the absence of *Hes1* and *Hes5*, cells infected with caNotch-AP sometimes formed large clusters (Figure 6A–F), but more often they were present as single cells (Figure 6J and K) or small clusters of 2–10 cells (Figure 6G–I; Table I). This is in sharp contrast to the caNotch-AP-infected cells in the wild-type, *Hes1*-null or *Hes5*-null background, which always formed large clusters (see Figures 1K, 4D and 5C). In addition, many of the caNotch-AP-infected cells expressed MAP2 and extended neurites in the absence of *Hes1* and *Hes5* (Figure 6; Table I). Thus, these virus-infected cells differentiated into neurons. Furthermore, the $MAP2^+$ ratio of the caNotch-expressing cells in the *Hes1-Hes5* double-null background was comparable to that of C-AP-infected cells in the same background, and wild-type (Table I). These results demonstrate that caNotch failed to inhibit neuronal differentiation in the absence of both *Hes1* and *Hes5*. However, neurite extension of the caNotch-AP-infected cells that expressed MAP2 was still poor, particularly in large clusters (Figure 6A–F), suggesting that caNotch can still partially inhibit neuronal differentiation independently of *Hes1* and *Hes5*. Nonetheless, these data demonstrate that *Hes1* and *Hes5* are essential for the full activity of Notch to inhibit neuronal differentiation.

Because the differentiation state of *Hes1-Hes5* double-mutant precursor cells could be more advanced than that

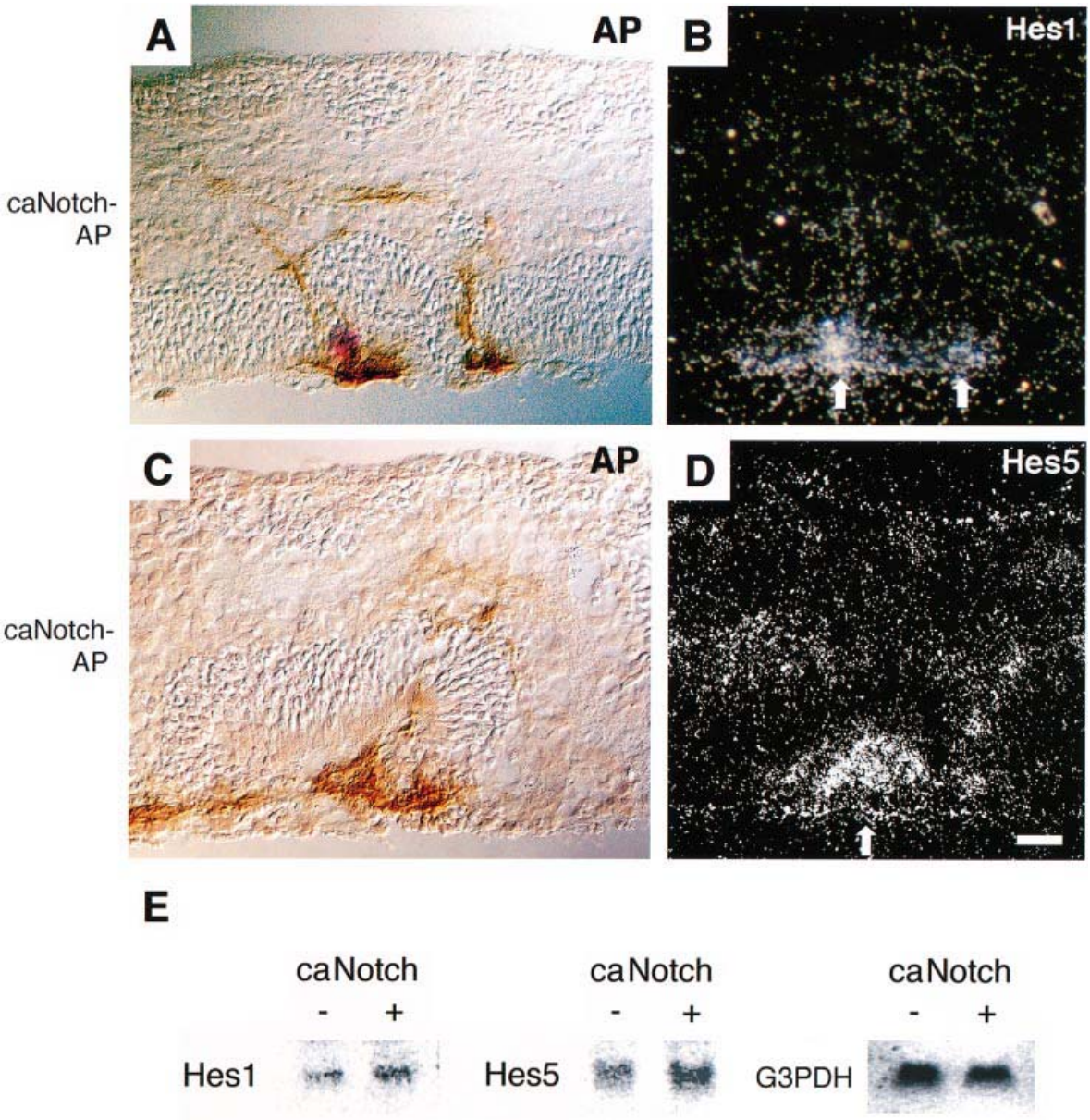

Fig. 2. Endogenous *Hes1* and *Hes5* expression in caNotch-AP-infected cells. The retinal explants (A–D) and neural precursor cells (E) prepared from wild-type embryos were infected with caNotch-AP. (**A**–**D**) Sections of the explants were chemically stained for AP expression [(A) and (C), brown], and the next sections were examined for *Hes1* (B) and *Hes5* expression (D) by *in situ* hybridization. The virus-infected cells expressed both *Hes1* and *Hes5* (arrows). Scale bar, 25 μm. (**E**) Total RNA (20 μg) was prepared from neural precursor cell cultures infected with caNotch-AP (+) or C-AP (–), and subjected to Northern blot analysis to determine *Hes* expression. Both *Hes1* and *Hes5* expression was weakly induced by caNotch. Glyceraldehyde-3 phosphate dehydrogenase (G3PDH) cDNA was used as a control probe.

of the wild-type, *Hes1*-null or *Hes5*-null cells, it is possible that E10.5 in the former background may correspond to a later stage of the latter backgrounds. Thus, the failure of caNotch to inhibit differentiation could be the result of the inability of caNotch to revert the cells from a more differentiated state to an undifferentiated state, rather than the inability of caNotch to prevent differentiation. We therefore infected wild-type neural precursor cells at E12.5 and later, with caNotch-AP virus. However, none of the virus-infected cells differentiated into $MAP2^+$ neurons (data not shown), suggesting that caNotch can inhibit neuronal differentiation of wild-type cells even at later stages. In order to show decisively that *Hes1-Hes5* double-mutant precursor cells did not advance to the stage at which neuronal differentiation cannot be prevented, we made retrovirus that carried *Hes1* and *green fluorescent protein* (*GFP*). Wild-type cells infected with this virus formed clusters without any neurite extension (Figure 7A and C, GFP^+) and did not express MAP2 (Figure 7B and C). Similarly, *Hes1-Hes5* double-null cells infected with the *Hes1–GFP* virus formed clusters and did not extend neurites (Figure 7D and F, GFP^+). In addition, none of the virus-infected colonies (out of >100 GFP^+ colonies) expressed MAP2 (Figure 7E and F), suggesting that these virus-infected cells were inhibited from differentiating into neurons. Thus, *Hes1-Hes5* double-mutant precursor cells did not advance to the stage at which neuronal differentiation cannot be prevented. These results strongly support the conclusion that the failure of caNotch to inhibit neuronal differentiation was not due to the advancement of neuronal differentiation at the time of viral infection, but rather due to a lack of essential effectors.

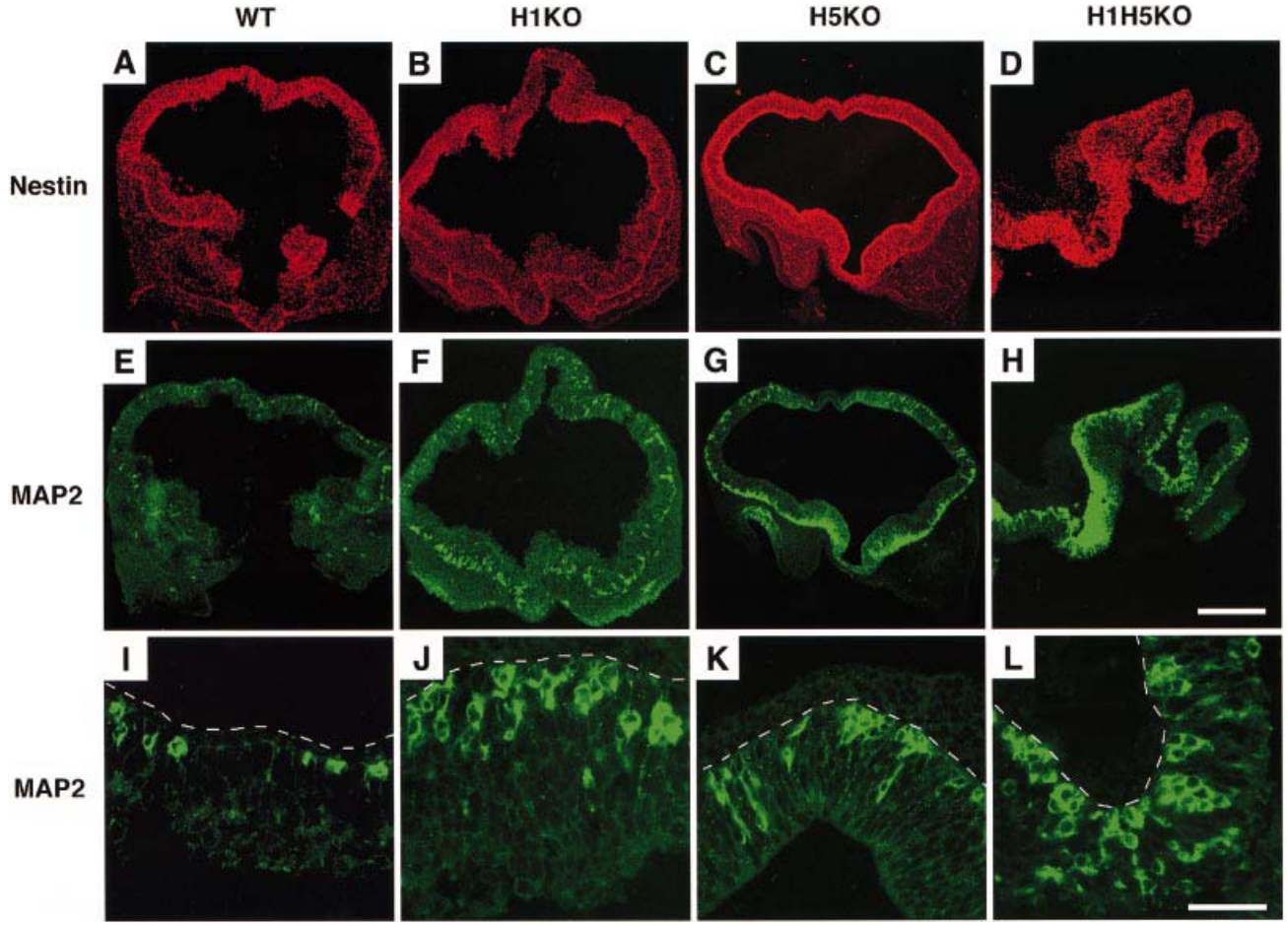

Fig. 3. The forebrains of wild-type and *Hes1*-, *Hes5*- and double-mutant mice at E10.5. Frontal sections of wild-type and mutant brains were examined immunohistochemically with anti-nestin (A–D) and anti-MAP2 antibodies (E–L). (**A**–**D**) There were many nestin$^+$ neural precursor cells in wild-type and mutant brains. (**E**–**H**) MAP2$^+$ neurons were present in the telencephalon (the upper half) and increased in number in the mutant brains. (**I**–**L**) A higher magnification of the medial part of the telencephalon. The dotted line indicates the pial surface. (I) MAP2$^+$ neurons appeared at the pial surface. (J–L) MAP2$^+$ neurons appeared at a greater density. Some cells prematurely expressed MAP2 before reaching the pial surface. Note that the double-mutant brain was smaller and deformed (D, H). Scale bars, 200 μm (A–H); 40 μm (I–L).

To determine whether the pathway from caNotch to activation of *Hes1* promoter is intact in *Hes1-Hes5* double-mutant cells, the luciferase reporter gene under the control of *Hes1* promoter was introduced into the wild-type and double-mutant cells, and activation of the promoter activity by caNotch was examined. Co-transfection of the caNotch expression vector exhibited ~20-fold upregulation of the *Hes1* promoter activity in both wild-type and *Hes1-Hes5* double-null cells (Figure 7G). These results demonstrate that the pathway from caNotch to activation of *Hes1* promoter is intact in *Hes1-Hes5* double-mutant cells.

Discussion

Hes1 and Hes5 as essential components for the Notch pathway

Activation of the membrane protein Notch inhibits cellular differentiation, and this inhibition is important for maintenance of multipotent progenitors and generation of cell-type diversity. In this study, we show that caNotch induces the endogenous expression of *Hes1* and *Hes5*, and that in the absence of both *Hes* genes, it fails to inhibit neuronal differentiation. These results demonstrate that *Hes1* and *Hes5* function as essential Notch effectors in regulation of neuronal differentiation.

The failure of caNotch to inhibit differentiation could be the result of the inability of caNotch to revert the cells from a more differentiated state to an undifferentiated state, because of premature neuronal differentiation in the double mutants rather than the inability of caNotch to prevent differentiation. However, we showed that differentiation of the double-mutant cells were inhibited by re-introduction of *Hes1*, thus indicating that these mutant cells did not advance to the stage when neuronal differentiation cannot be prevented. In addition, these data demonstrated that *Hes1* can substitute for the inhibition of differentiation, thus strongly supporting the conclusion that *Hes* genes are essential Notch effectors for inhibition of neuronal differentiation. We also showed that caNotch can upregulate *Hes1* promoter activity in *Hes1-Hes5* double-mutant cells, thus excluding the possibility that the pathway downstream of Notch, besides *Hes* genes, is affected.

Apparently, *Hes1* and *Hes5* are functionally redundant, and when either gene is present, Notch can still function. Inactivation of either *Hes1* or *Hes5* leads to premature neuronal differentiation, and therefore both genes have a

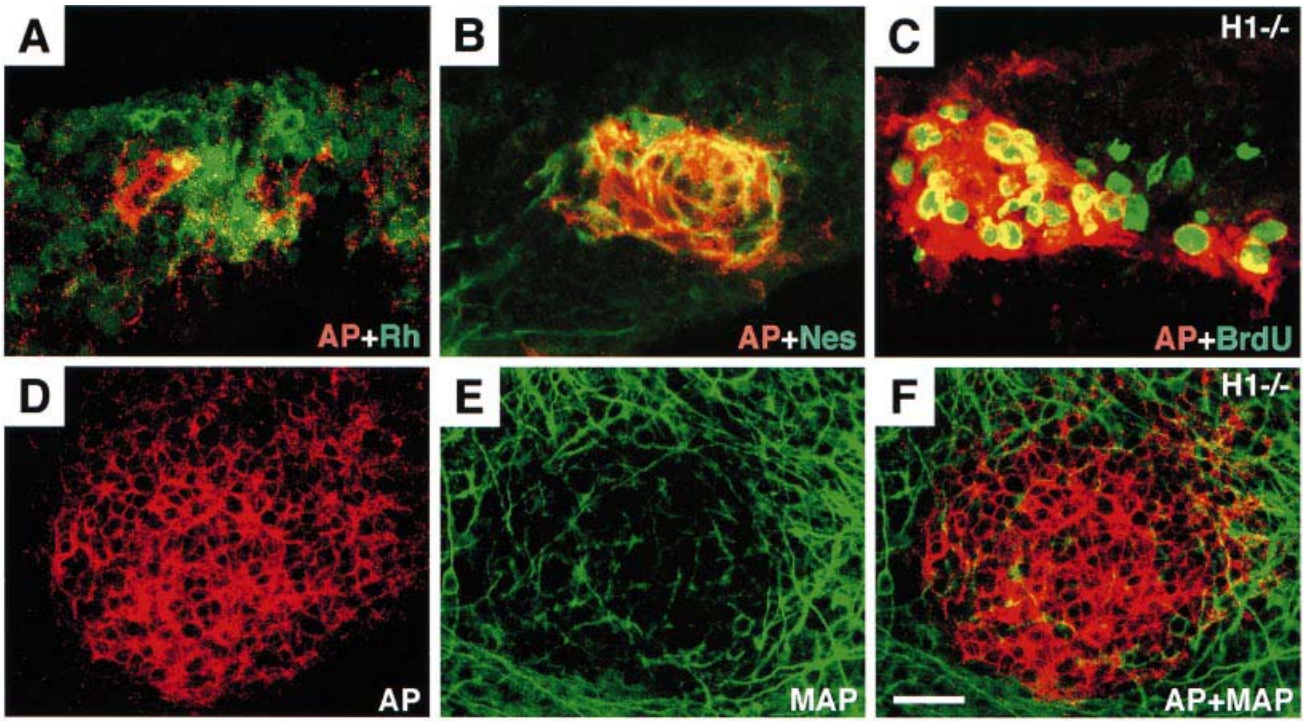

Fig. 4. *Hes1*-null cultures infected with caNotch-AP. (**A**–**C**) Retinal explants from *Hes1*-null embryos were infected with caNotch-AP, and for the last 2 days of culture BrdU was added. The explants were stained with antibodies against AP [(A)–(C), red], Rh [(A), green], nestin [(B), green], and BrdU [(C), green]. caNotch-expressing *Hes1*-null cells did not express rhodopsin, but expressed the progenitor-specific marker nestin and incorporated BrdU, indicating that these cells did not differentiate into retinal neurons. Note that the laminar structure was disrupted in the absence of *Hes1*. (**D**–**F**) *Hes1*-null neural precursor cell cultures infected with caNotch-AP were stained with antibodies against AP [(D) and (F), red] and MAP2 [(E) and (F), green]. Note that the virus-infected cells and the $MAP2^+$ cells were segregated. Scale bar, 25 μm.

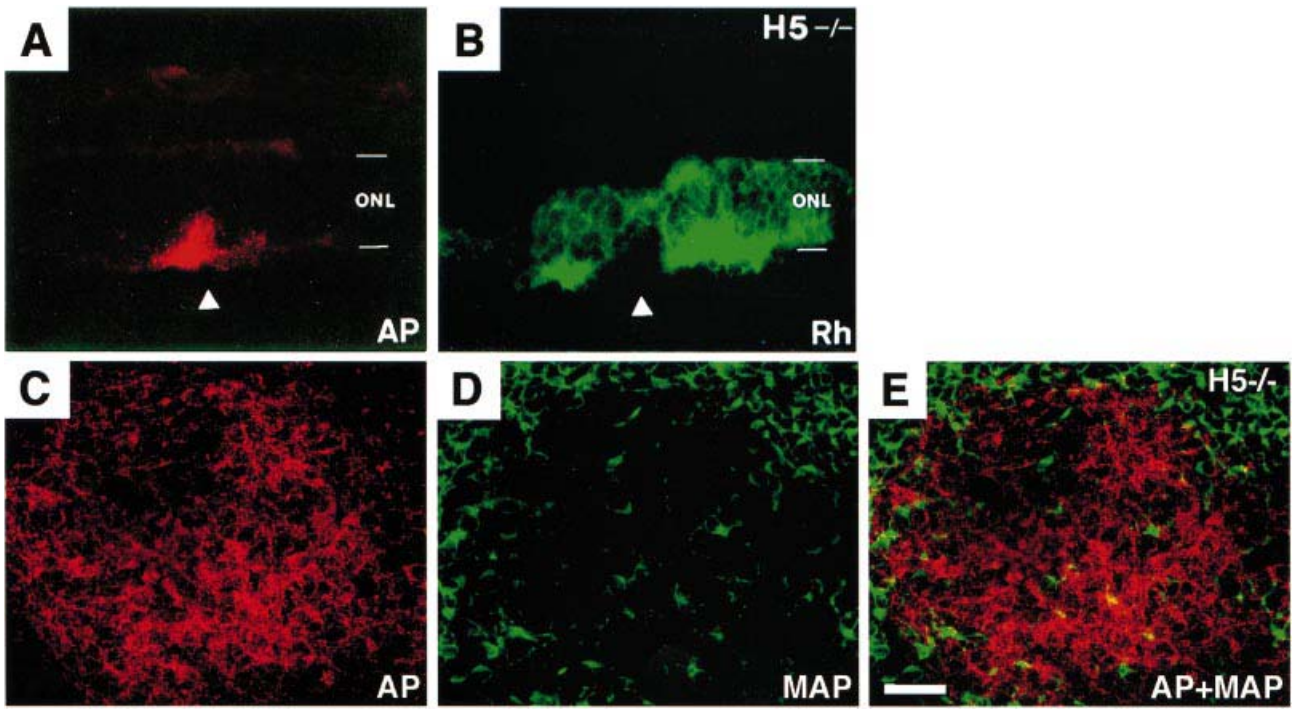

Fig. 5. *Hes5*-null cultures infected with caNotch-AP. (A and B) *Hes5*-null retinal explant cultures infected with caNotch-AP were stained with antibodies against AP (**A**) and Rh (**B**). The virus-infected *Hes5*-null cells formed a cluster and did not express Rh (arrowhead). (**C**–**E**) *Hes5*-null neural precursor cell cultures infected with caNotch-AP were stained with antibodies against AP [(C) and (E), red] and MAP2 [(D) and (E), green]. Note that the virus-infected cells and $MAP2^+$ cells were segregated. Scale bar, 25 μm.

similar function: inhibition of premature neurogenesis. However, these two genes do not seem to be functional equivalents, since the severity of the mutant phenotypes is totally different: *Hes1*-null mutation leads to embryonic or postnatal lethality with neural tube and eye defects (Ishibashi *et al*., 1995; Tomita *et al*., 1996a), whereas

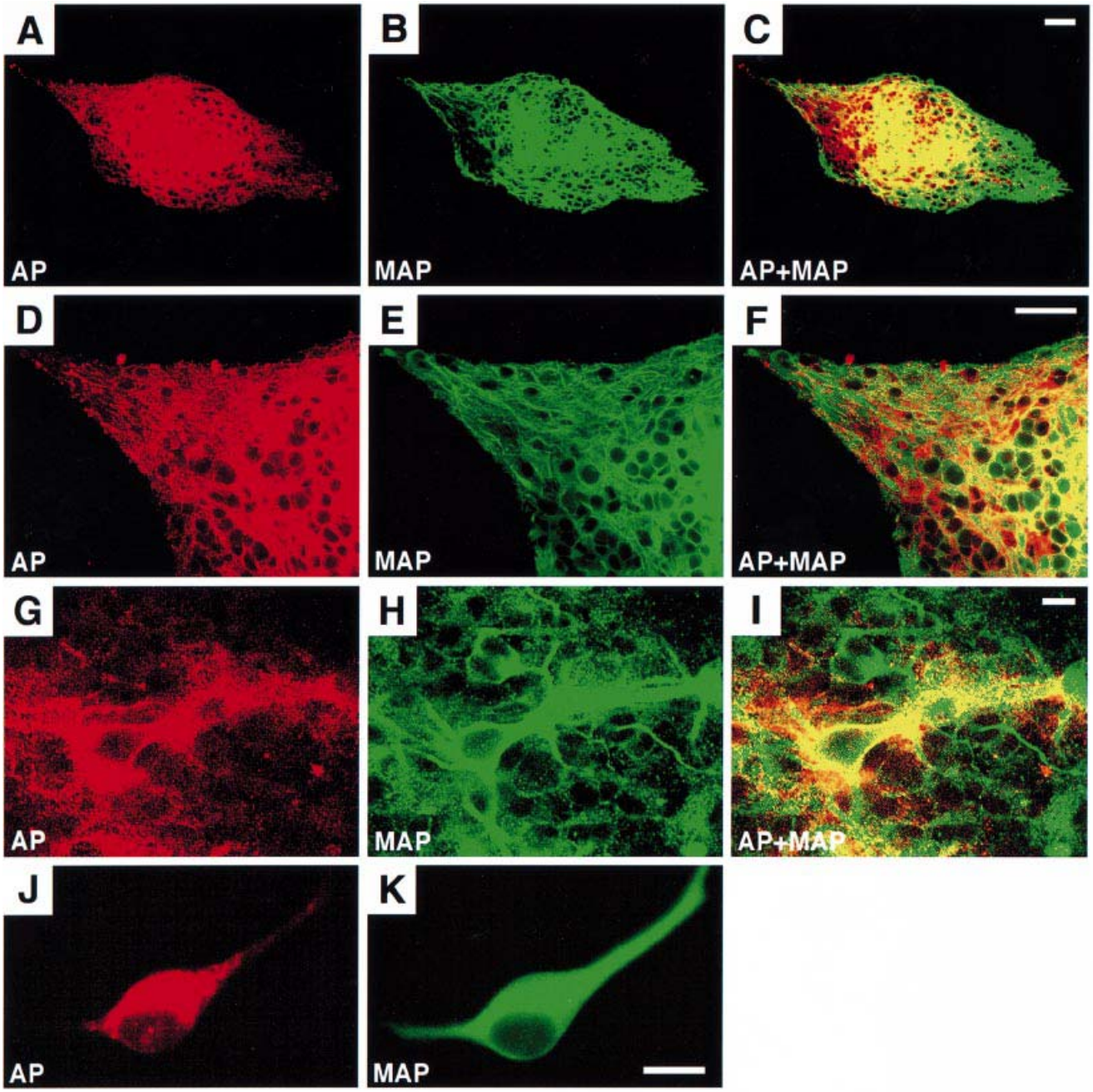

Fig. 6. *Hes1-Hes5* double-null neural cells infected with caNotch-AP. *Hes1-Hes5* double-null neural precursor cell cultures infected with caNotch-AP were stained with antibodies against AP (A, C, D, F, G, I, J, red or yellow) and MAP2 (B, C, E, F, H, I, K, green or yellow). (**A**–**C**) These virus-infected cells formed a large cluster and expressed MAP2. (**D**–**F**) A higher magnification of (A–C). These virus-infected cells extended rather poor neurites. (**G**–**I**) A cluster of two cells infected with caNotch-AP extended neurites and expressed MAP2. (**J**, **K**) An isolated single cell infected with caNotch-AP differentiated into a $MAP2^{+}$ neuron in the absence of *Hes1* and *Hes5*. Scale bars, 25 μm (A–F); 5 μm (G–K).

Hes5-null mice were morphologically normal in spite of premature neuronal differentiation at E10.5. Thus, the phenotypes of *Hes5* mutation seem to be mostly compensated by other genes, but those of *Hes1* mutation do not. Therefore, *Hes1* rather than *Hes5* may be a major component for the Notch pathway. Another possibility is that *Hes1* has an additional function beside being effector of Notch signaling. This is suggested by the fact that *Hes1* expression is controlled by additional upstream regulatory regions besides the RBP-J sites (Takebayashi *et al*., 1994; Issack and Ziff, 1998).

The pathway from Notch activation to inhibition of differentiation

The mammalian *achaete-scute* homologue *Mash1*, which regulates differentiation of autonomic, olfactory, retinal and telencephalic neurons (Guillemot *et al*., 1993; Sommer *et al*., 1995; Tomita *et al*., 1996b; Cau *et al*., 1997; Casarosa *et al*., 1999), is one of the target genes for the Notch–Hes pathway, since inactivation of *Notch1*, *RBP-J* or *Hes1* ectopically upregulates *Mash1* expression (Ishibashi *et al*., 1995; de la Pompa *et al*., 1997). It is shown that Hes1 binds directly to the *Mash1* promoter

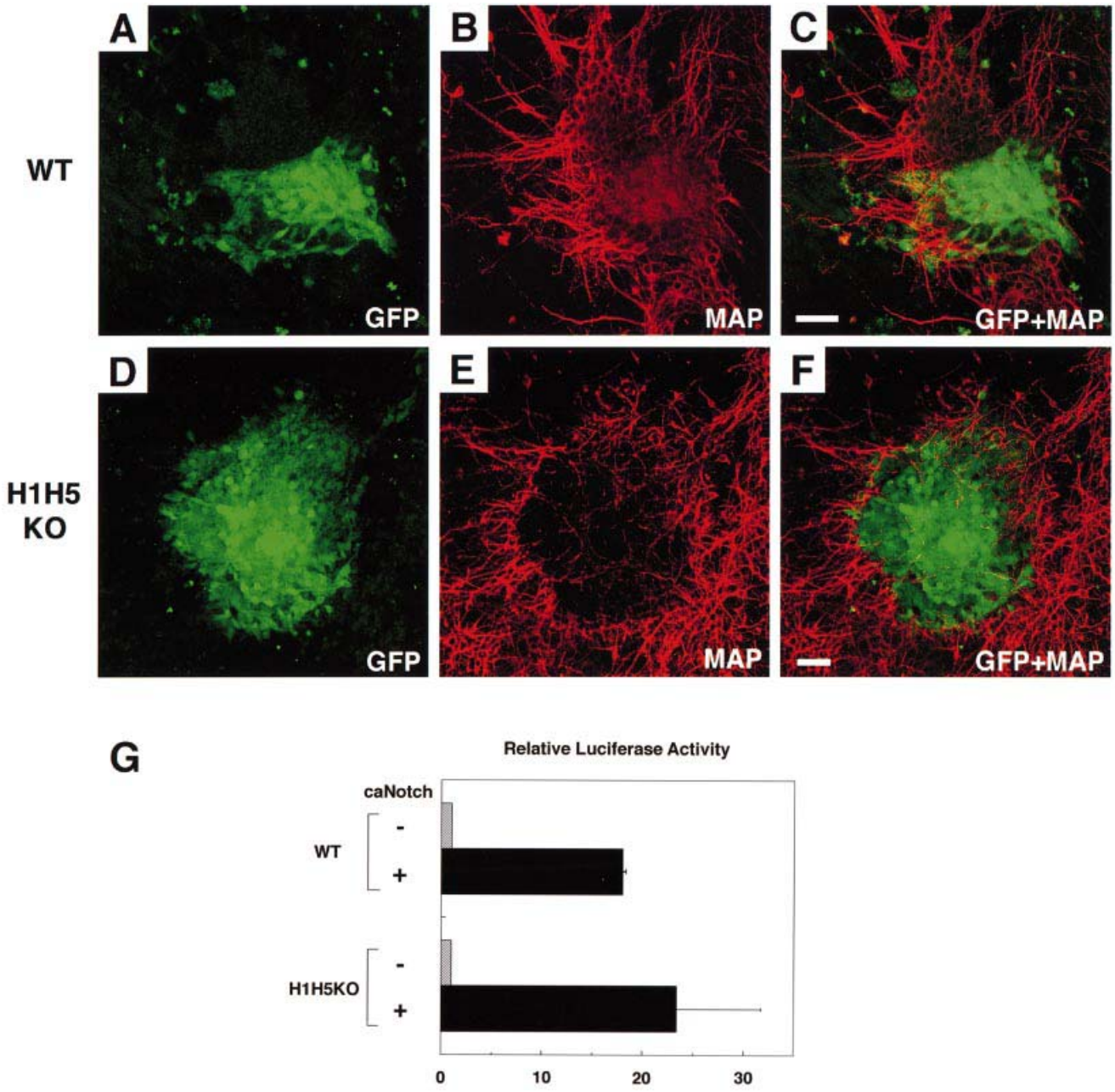

Fig. 7. *Hes1* virus infection and *Hes1* promoter activity in *Hes1-Hes5* double-null neural cells. (**A**–**C**) Wild-type cells were infected with *Hes1–GFP*-transducing retrovirus. The virus-infected cells [(A) and (C), green] formed a cluster without any neurite extension and were negative for MAP2 expression [(B) and (C), red]. Thus, *Hes1* inhibited neuronal differentiation. (**D**–**F**) *Hes1-Hes5* double-null neural precursor cells were infected with *Hes1–GFP*-transducing retrovirus. The virus-infected cells [(D) and (F), green] formed a cluster and were negative for MAP2 expression [(E) and (F), red]. Thus, neuronal differentiation of *Hes1-Hes5* double-null cells was blocked by re-introduction of *Hes1*. Scale bar, 25 μm. (**G**) The luciferase reporter gene under the control of *Hes1* promoter was transfected into wild-type and *Hes1-Hes5* double-null neural cells. The caNotch expression vector (+) or control vector (–) was also co-transfected. caNotch showed ~20-fold upregulation of the *Hes1* promoter activity in both wild-type and *Hes1-Hes5* double-null cells. Each value with a standard error is the average of at least three independent experiments.

and represses *Mash1* expression (Chen *et al*., 1997). Thus, *Drosophila hairy/Enhancer-of-split*, which antagonize *achaete*, and mammalian *Hes1*, which represses *Mash1*, have been well conserved functionally as well as structurally during evolution. Other target genes would include *Math1*, which is essential for generation of cerebellar granule cells and is functionally antagonized by Hes1 (Akazawa *et al*., 1995; Ben-Arie *et al*., 1997), and *Neurogenins*, which regulate neuronal determination of cranial ganglions and are repressed by the Notch signaling (Fode *et al*., 1998; Ma *et al*., 1998). Thus, the neuronal determination and differentiation in mammals may be regulated by the pathway *Notch*→*RBP-J*→*Hes1*/*Hes5*⊣*Mash1*/*Math1*/*Neurogenins*.

It was shown recently that *Notch1* is specifically expressed by ependymal cells, neural stem cells in the adult central nervous system (CNS) (Johansson *et al*., 1999). These cells divide asymmetrically to generate two different daughter cells, a differentiating cell and a stem cell, and only the latter cell maintains *Notch1* expression.

We showed previously that cells persistently expressing *Hes1* with retrovirus become ependymal cells in the CNS (Ishibashi *et al*., 1994). Thus, the Notch–Hes pathway may play an important role in maintenance of neural stem cells in the CNS.

Although our data show that *Hes1* and *Hes5* are essential for the Notch pathway, there seem to be other molecules that mediate Notch signaling, since caNotch can still partially inhibit neuronal differentiation in the absence of *Hes1* and *Hes5*. The notion that the two *Hes* genes are essential but not sufficient for the Notch pathway is also supported by the phenotypes of *Hes1-Hes5* double-null embryos, which are less severe than those of *Notch1*-null embryos; whereas *Notch1*-null embryos do not survive beyond E11.5, *Hes1-Hes5* double-null embryos survive up to E12 (Swiatek *et al*., 1994; Conlon *et al*., 1995; de la Pompa *et al*., 1997). Recent data show that Notch can inhibit differentiation genes independently of *RBP-J* and *Hes*. Notch can block the muscle-cell fusion process of C2C12 myoblasts independently of *RBP-J* and *Hes1* (Shawber *et al*., 1996). It was also shown that the Notch-interacting protein Deltex can inhibit the bHLH factor E47 independently of RBP-J (Matsuno *et al*., 1998; Ordentlich *et al*., 1998). Thus, these alternative pathways may compensate for *Hes1* and *Hes5* deficiency in the Notch signaling.

We provide evidence that Notch and Hes constitute a major pathway in maintaining of the undifferentiated state. Further characterization of this pathway would help to understand how the timing of cellular differentiation is determined and how the cell-type diversity is generated from stem cells.

Materials and methods

Preparation of retrovirus

For construction of the caNotch-AP virus, the *Ssp*I–*Xho*I fragment of mouse *Notch1* cDNA (from nucleotide residues 5188–6655), tagged with the Flag sequence at the *Ssp*I site (a gift from Masashi Kawaichi and Gerry Weinmaster), was cloned into the *Eco*RI site of the retroviral vector 1726 AP w/o neo (kindly provided by Jane Burns). For construction of the C-AP virus, the *Nae*I–*Sac*II region (from nucleotide residues 5330–6338) was deleted from the caNotch-AP construct. The structure of *Hes1–GFP*-transducing retrovirus will be described elsewhere (M.Hojo, T.Ohtsuka and R.Kageyama, unpublished data). The DNAs were transfected with LipofectAMINE (Gibco-BRL) into ψ2mp34 (Yoshimatsu *et al*., 1998), an ecotropic packaging cell line (a gift from Kazuhiro Ikenaka). The supernatant was collected 2 days later and concentrated with Centriprep 100 (Amicon), as described previously (Ishibashi *et al*., 1994; Tsuda *et al*., 1998).

Retinal explant culture

The retinal explant culture and retroviral infection were performed as described previously (Sparrow *et al*., 1990; Tomita *et al*., 1996a) except that retinas were isolated at E16.5. Two weeks after infection, retinas were fixed and embedded in OCT compound (Miles), and cryosections were made. The sections were examined by either immunohistochemistry or chemical staining.

Neural precursor cell culture

The primary culture of neural precursor cells was performed as described previously (Ishibashi *et al*., 1994; Miyata and Ogawa, 1994). The uterus containing E10.5 mouse embryos was excised and incubated in minimal essential medium (MEM) for 1 h. Then, the embryos were isolated and their heads were excised. With fine forceps, the epidermis, mesenchyme and meninges were removed. The neuroepithelium of the forebrain was transferred into fresh phosphate-buffered saline (PBS) and partially dissociated by pipetting. The cells were resuspended in the medium [1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12 containing 5% fetal bovine serum (FBS), 25 μg/ml of transferrin, 15 μg/ml of insulin, 20 nM progesterone, 30 nM sodium selenite, 1.7 ng/ml of basic fibroblast growth factor (bFGF), 3.3 ng/ml of epidermal growth factor (EGF), 10 ng/ml of nerve growth factor (NGF) and 10 ng/ml of cholera toxin] and plated onto a tissue culture dish. The next day, non-adhesive cells were collected and replated onto polyethylenimine and fibronectin-coated chamber slides (Lab-Tek) at a density of 3×10^4. After the cells were attached to the slides, virus solution with 8 μg/ml of polybrene was added to the culture. Three hours later, the solution was aspirated and fresh medium was added. After 2 weeks of culture, the cells were fixed and examined by immunohistochemistry.

Generation and genotyping of Hes1- and Hes5-null mice

For genotyping of *Hes1*-null mice, the 0.9-kb *Bam*HI–*Pvu*II fragment was used as a probe, as described previously (Ishibashi *et al*., 1995). This probe detected a 12-kb wild-type band and a 2.3-kb mutant band.

Hes5-null mice were generated by targeted disruption of the *Hes5* locus, which replaced the first two exons encoding the bHLH domain (Takebayashi *et al*., 1995) with a neomycin-resistance expression cassette. The detailed strategy will be described elsewhere (E.Cau, G.Gradwohl, R.Kageyama and F.Guillemot, unpublished data). The genetic background of the mutant mice was 129Sv×ICR. For genotyping of *Hes5*-null mice, a 0.9-kb *Sma*I–*Bam*HI fragment was used as a probe. This probe detected a 7-kb wild-type band and a 4-kb mutant band.

Hes1-Hes5 double-null embryos were obtained by crossing *Hes1*$^{+/-}$-*Hes5*$^{-/-}$ or *Hes1*$^{+/-}$-*Hes5*$^{+/-}$ mice.

In situ hybridization and Northern blot analysis

In situ hybridization and Northern blot experiments were carried out as previously described (Akazawa *et al*., 1992; Sasai *et al*., 1992). ^{35}S-labeled cRNA was used as a probe for *in situ* hybridization.

Immunohistochemistry and immunocytochemistry

Fixed samples were rinsed in PBS and blocked with 2% normal goat or donkey serum. Primary antibodies used were as follows: rabbit anti-human placental AP (1:10; Nichirei), mouse anti-human placental AP (1:100; Chemicon), anti-GFP (1:500; Clontech), mouse anti-Flag (1:3000; M2, Kodak), rabbit anti-rhodopsin (1:2000; LSL), mouse anti-protein kinase C (1:100; Amersham), mouse anti-calbindin (1:250; Sigma) and mouse anti-MAP2 antibodies (1:500; Clone HM-2, Sigma). To detect these antibodies, FITC-conjugated goat anti-mouse antibody (1:100; Cappel), FITC-conjugated donkey anti-goat antibody (1:100; Chemicon), biotinylated anti-rabbit antibody (1:200; Vector), and Texas Red avidin D (1:1000; Vector) were used.

For BrdU staining, BrdU (2 μM) was added to the culture for the last 2 days and stained with anti-BrdU antibody (1:5; Becton Dickinson).

Luciferase reporter assay

Transient transfection with the *Hes1* promoter–luciferase vector and caNotch expression vector was performed and the luciferase activities were determined as described previously (Nishimura *et al*., 1998).

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